RECENT DEVELOPMENTS IN MICROSCOPY*

BY

A. S. McFARLANE

National Institute for Medical Research, London

In 1873 the German physicist, Abbe, published his theory of the microscope objective. It is summed up in a relatively simple equation, namely :

$$d = \frac{\lambda}{2\ N.\ \text{Sin.}\ \alpha}$$

Where d is the smallest distance apart at which two particles can just be discerned as separate, λ is the wavelength of the light, N is the refractive index of the immersion oil, and α is the half angle subtended by the lens at the object.

In general, microscope objective lenses are designed for use with particular values of N and α and the product $N \sin \alpha$ is therefore given a special name, the Numerical Aperture. Since the sine of an angle cannot exceed unity the N.A. cannot exceed the refractive index of the medium for which the lens is designed, e.g. 1·60 in the case of naphthaline bromide. It is further obvious that once this upper limit of the N.A. is reached the only way in which resolution can be further increased is by employing light of smaller wavelength.

Serious efforts in this direction began in 1904 when Kohler and von Rohr in Germany produced quartz-fluorite objectives designed for use with ultra-violet light of 275 mμ wavelength. Thus, by approximately halving the effective wavelength of the light used, they were able to double the distance d or the resolution. Since the object is invisible their eye-piece was fitted with a uranium glass fluorescent screen which enabled the field to be searched and focused in a crude way. Subsequently the eye-piece was removed and a photographic ocular and plate substituted. Barnard, who worked at the National Institute for Medical Research at Hampstead, purchased such an outfit from the Germans in 1913 and found it to have serious technical defects. These he proceeded to correct ; for example, the low ultra-violet intensity of the German light-source was increased by replacing the induction coil of the spark by a transformer, and the fluorescent searcher was replaced by visual focusing. In the latter procedure visible light from the same spark is used to focus the object, and thereafter ultra-violet light is substituted, the photographic plate put in position, and the fine focusing control moved by a predetermined amount to adjust for the different focal lengths of the optical system at the different wavelengths. It is perhaps needless to say that these adjustments must be made with great mechanical precision, and Barnard, and his associate Welch, designed very beautiful devices for this purpose.

Parenthetically, may I say that Barnard, who died a few months ago, was yet another outstanding amateur to adorn British science. He never acquired a degree, although he was elected a Fellow of the Royal Society, and throughout his active life at Hampstead he also supervised a business in London.

One fundamental difficulty remained however, namely the intrinsic lack of contrast in biological materials. This is particularly evident in small objects and is a difficulty which I feel sure many of you will have experienced in detecting spirochaetes. The solution in Barnard's case was to use dark-ground illumination ; for this purpose he collaborated with the optical firm of Beck, and they produced the Duplex condenser in 1924. This functions alternatively as a cardioid condenser for illuminating the object obliquely, and as a conventional condenser for direct illumination, the change-over being effected merely by changing stops. Beck also built, to Barnard's specification, a micrometer screw for fine focusing which remains today a model of precision engineering, and a quartz-fluorite objective of N.A. 1·20 for use with light of wavelength 275 mμ. The only significant further improvement to the Beck-Barnard microscope was made in 1934, when Zeiss built for Barnard an objective of N.A. 1·25, corrected for the 257 mμ line.

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It is interesting to note that while this valuable tool was in steady use in biological research at Hampstead for some twenty years it received little or no attention elsewhere. It was presumably too expensive and too complicated for the smaller biological laboratory, and the gain in resolution seems not to have impressed non-biological users. However, the advent of atomic fission brought about a change. There are grounds for believing that early radiation damage to the health of uranium workers may be diagnosed from ultra-violet micrographs of leucocytes even before a significant leucopenia develops. I do not know how significant this observation is, but it seems to have provided the stimulus required for the instrument to be manufactured. The firm of Cooke, Troughton, and Simms of York have now made six instruments embodying the best features of the Hampstead one and several original features.

At this point we may profitably examine a little more closely the nature of the biological object. Ideally the refracting microscope—whether visual or ultra-violet—should be uniform in its refractive properties and vary only in colour. In such a case the rays of light from each point source will radiate in all directions and vary only in intensity. This, of course, is never quite attained in practice. At one point the cell may be watery and of low refractive index and at another (e.g. in the nucleus) it may be dehydrated and therefore of high refractive index. Refraction gradients which exist in the intervening regions bend the incident light rays, and if the gradient is steep the emergent rays may fail to reach the eye-piece at all. Thus that part of the cell in which the light was deviated appears dark in the image, and we cannot say whether the blackness is due to refraction, or absorption, or both.

In general, thin biological objects do not show refraction gradients of this order. Unfortunately they also show little or no colour contrast, and the images, however highly resolved, are frequently too faint to be seen or photographed—at least with visible light, and artificial contrast has to be created by differential staining. With ultra-violet light the position is more satisfactory, since all proteins, and especially the nucleoproteins, absorb highly in this region. Thus, in general, contrast as well as resolution is better with ultra-violet than with visible light. It is important to note that the absorption of biological materials can be very different at 275 mμ and 257 mμ and that therefore photographs of the same object with light of both wavelengths may not look the same. Differential photography of this kind in the ultra-violet has been developed by Caspersson and his Swedish school into a highly effective cytological technique for localizing nucleic acids in the cell.

Proteins also fluoresce in ultra-violet light and therefore the best technique for smaller biological objects is to make them self-luminous by oblique illumination with this kind of radiation. This is the method of choice, for example, with suspensions of viruses. As a rule it gives information only about the size and shape of the organisms. Internal detail is largely confused by the generalized light scattering and this, of course, is particularly evident with thick specimens, such as histological sections or tissue culture cells. In these cases direct ultra-violet or visual illumination is better, although still far from perfect because of the low colour contrast.

(Here Dr. McFarlane showed slides of the microscope pointing out the precision focusing device, dual illumination arrangements, and various other features. He also showed several pictures taken by ultra-violet light, including slides of *B. anthracis*, *B. megatherium*, *T. pallidum*, and the Canary Pox virus.)

In recent years, however, a new development has occurred, the technical details of which I will not attempt to enter into. It will suffice to say that so-called ' phase plates ' are introduced into the condenser and objective lenses of the ordinary microscope, and that these have the effect of absorbing light which has suffered deviation however small in its passage through the object. Thus a refraction gradient in the object becomes evident as light absorption in the image. Contrast is thus created over and above that due to the intrinsic colour of the object. It is, of course, somewhat difficult to dissociate the two in the final image, and it is probably true to say that the interpretation of the phase-contrast image is still a matter of controversy. There is, however, no controversy about the fact that internal detail in cells can now be seen with the phase-contrast microscope which hitherto could not be seen at all. There is no gain—in fact there is a slight loss—of resolution on introducing phase plates. However, to obtain the same degree of contrast in the ordinary microscope would necessarily involve a heavy sacrifice of resolution so that for the same degree of contrast the resolution of the phase microscope is higher than that of the ordinary microscope.

The idea of phase contrast is due in the first instance to the Dutch physicist, Zernicke, and its practical development is due to the firm of Zeiss. Recently, however, Cooke, Troughton, and Simms, in collaboration with my colleague Mr. Smiles, have produced the first phase-contrast microscope for ultra-violet light. It is too early to predict its success, but at least it is clear that the visual phase-contrast searcher which they use has become a permanent accessory of the Beck-Barnard ultra-violet

microscope. It gives better results with thick objects than either dark-ground or direct illumination.

A still more recent development is exclusively British. Burch in Bristol has perfected the reflecting microscope. Again I cannot enter into details but the principle is not new. Light on passing through a lens is refracted and at the same time dispersed, i.e., if the light is heterochromatic. Getting rid of this dispersion is one of the main problems which the designer of a microscope objective has to face. On the other hand, light which is reflected from a curved surface will focus in a more or less magnified image of the object but without dispersion, and, therefore, with no defects in the image due to chromatic aberration. The reflecting lens is therefore achromatic but still suffers from defects of spherical aberration, which can only be corrected by patient handworking of the reflecting surface into a complicated paraboloidal shape. Burch has done this and has produced an achromatic objective of N.A. 0·9. Of course, this is ideal for use with ultra-violet light since it gets rid of the difficult problem of colour correction in ultra-violet objectives and permits of visual focusing. Furthermore, it has the valuable property of a very long working distance—instead of millimetres this is nearly an inch—which makes it quite unequalled for use in microdissection. The question at the moment is whether the reflecting microscope can be manufactured. Burch thinks that it can and he is at present working on a three-year plan to prove his point. Note, however, before we leave this matter, that neither the phase-contrast nor the reflecting microscope improves on the limit of resolution set by the wavelength of the light used. This brings us to the electron microscope—quite the most surprising development of all.

It happens that a beam of electrons can be visualized in the manner of modern physics, either as a stream of discrete charged particles, or as an electromagnetic vibration equivalent in all respects to a beam of light but of infinitely smaller wavelength. In fact, the wavelength depends on the speed of the electrons and this in turn can be manipulated by altering the positive voltage on the electrode or plate to which they are attracted. If the accelerating voltage is 50,000 volts the equivalent wavelength is only 0·005 mμ, which implies according to Abbe's formula that resolutions are possible which are higher by a factor of 10,000 than those given by the light microscope. The electrons are allowed to pass through a hole in the accelerating plate and thereafter they are condensed on the specimen which is imaged by successive objective and projector lenses on a photographic plate or fluorescent screen. The lenses, of course, are magnetic ones, the focal lengths of which can be altered continuously by electrical means. The one serious difficulty is that electrons are easily absorbed in air and therefore the whole electron optical system including the specimen must be maintained in a high vacuum. Only perfectly dry materials can be examined, and elaborate devices are required for changing specimens and photographic plates with the introduction of minimal amounts of air. Pumps which work continuously get rid of this air inside a minute.

In the course of drying, most biological objects shrink and distort in some degree, even after treatment with histological fixatives of the type of osmic acid. To this extent the images are unreliable, but it would be a mistake to assume, as has been done, that they are incapable of giving useful information.

For example, in the field of viruses shrinkage is known to be in the region of 20 per cent., and distortion negligible. The agent of small pox is seen to have a brick-like shape which it shares with the agents of fowl pox, infectious ectromelia, molluscum contagiosum and others. The brick shape can be brought out very well by a technical trick which consists in depositing an electron opaque material like gold obliquely on the virus. The length of the shadow is a measure of the height of the brick and a very satisfactory relief effect is produced.

By contrast, influenza, herpes, and papilloma viruses, to name a few, are almost perfect spheres, although influenza appears to have also a filamentous form (Chu and others, 1949). The mosaic virus of the tobacco plant is rod-shaped, and on standing in the ice box for a few weeks the rods join up in pairs, the joint being as strong as the rest of the rods. There are plenty of physical measurements to confirm this aggregation and even to tell us that the mean size of the virus rods is doubled on standing, but only seeing the rods in the electron microscope has made it clear that the change is one of simple end-to-end aggregation in pairs.

Another instance in which the electron microscope has given us a unique kind of information concerns bacteriophage. The sizes of many of these bacterial viruses have been known from indirect physical measurements for some years. However, it is only when we come to see them that we discover that they have heads and tails.

Probably the supreme achievement in electron microscopy so far is shown in this picture. You will know that some of the plant virus molecules—and they are indeed nothing more than molecules of nuclear proteins endowed in some mysterious way with the propensity for self-duplication within

the host cell—crystallize readily into flat plates or cubes. Here is a picture of one face of such a crystal of the tobacco necrosis virus (Markham and others, 1948), and in a sense it is a fake since we are not actually looking at the crystal. A thin layer of collodion is placed on the crystal and stripped off again. It takes with it a permanent replica of the crystal surface which is faithful right down to molecular dimensions; but of course it has no intrinsic contrast. This is created artificially by gold shadowing, and we are here looking at the imprint on collodion of spherical molecules in a crystal lattice. We are thus able to observe the perfect regularity of a crystal structure which various physical methods in the past have told us existed. The molecules in this case are 24 mμ in diameter.

Gold shadowing, of course, can only give information about the surface contours, and to detect internal structure we have to overcome the difficulty of low intrinsic contrast—in this case low relative electron opacity—in biological materials. A method of overcoming this is to act on the cell with agents which selectively remove particular structures without damage to the cell as a whole. Only enzymes are sufficiently selective for this purpose, and fortunately a large and increasing number of these are becoming available; as, for example, the vaccinia virus after digestion with pepsin (Dawson and McFarlane, 1948).

Curiously enough, psittacosis virus is entirely unaffected by trypsin and pepsin (Barwell and others, 1949). The reason for this may be that it has a chitinous envelope which folds on drying and which is impervious to these enzymes. If this is so, psittacosis and the sister viruses of pneumonitis and lymphogranuloma venereum should probably be classified more closely with plant cells than with the protozoa.

The attack on bacterial structure with the electron microscope has only just begun. We start with the handicap that many bacteria are almost totally opaque to the electron beam, at least in some phases of the life cycle. In some cases the opacity resides largely in a tough capsule which can be detached by mechanical means (for example, *Staph. aureus*, Cooper and others, 1949). Flagella photograph extremely well, and appear to pass through the cell wall (for example, *Spirillum*, Van Iterson, 1947). In general the electron opacity of cocci, including the gonococcus, persists at all ages and the elucidation of internal structure must await the development of suitable cytochemical techniques. Many bacilli, especially in young cultures, are transparent to electrons and show internal bodies. Surprisingly, the tubercle bacillus (Lembke and Ruska, 1940) belongs to this group and shows no signs of the waxy envelope which for so long has been regarded as the site of its acid fastness. The inclusions seen in the avian bacillus are of a fatty nature.

In conclusion I regret that I have not more for you in the specifically venereal field. I believe, however, that techniques are available for substantial advances in this field, and all that is required is an enthusiastic venereologist to make use of them. He will need to employ a variety of techniques, including two or three forms of microscopy, since each has its own information to offer, and there must also be a considerable amount of microchemical investigation. The thorough elucidation of the nature of even one organism is a complicated matter requiring much time, but it can be done and will be done systematically during the next few years for the organisms in which you are particularly interested.

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